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16. ABSTRACT

The effects of simultaneous exposure to ozone (O_3) and sulfuric acid H_2SO, 0.23 m volume median diameter (VMD) and a single exposure to ultrafine (<0.1 m VMD) H_2SO_4 under various conditions were studied using the infectivity Mortality and the ciliary beating frequency model systems. A 3-h exposure to a combined aerosol of 196μg O_3/m^3 and 483 or 241μg H_2SO_4/m^3 significantly increased the susceptibility of mice to a laboratory-induced respiratory infection. However, exposure to 543μg ultrafine H_2SO_4/m^3 for 2 h or 365μg/m^3 2 h/d for 5 d did not significantly affect this parameter. Upper airway response, as measured by changes in hamster tracheal ciliary beating frequency, was not affected by either a 3-h combined exposure to 196μg O_3/m^3 and 847μg H_2SO_4/m^3 or a 2-h exposure to 458μg ultrafine H_2SO_4/m^3.

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PULMONARY HOST DEFENSE RESPONSES TO INHALATION OF SULFURIC ACID AND OZONE

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The effects of simultaneous exposure to ozone (O_3) and sulfuric acid [H_2SO_4, 0.23 μm volume median diameter (VMD)] and a single exposure to ultrafine (<0.1 μm VMD) H_2SO_4 under various conditions were studied using the infectivity/mortality and the ciliary beating frequency model systems. A 3-h exposure to a combined aerosol of 196 μg O_3/m^3 and 483 or 241 μg H_2SO_4/m^3 significantly increased the susceptibility of mice to a laboratory-induced respiratory infection. However, exposure to 543 μg ultrafine H_2SO_4/m^3 for 2 h or 365 μg/m^3 2 h/d for 5 d did not significantly affect this parameter. Upper airway response, as measured by changes in hamster tracheal ciliary beating frequency, was not affected by either a 3-h combined exposure to 196 μg O_3/m^3 and 847 μg H_2SO_4/m^3 or a 2-h exposure to 458 μg ultrafine H_2SO_4/m^3.

INTRODUCTION

Atmospheric sulfuric acid (H_2SO_4) is the result of gaseous and particulate emissions from mobile and stationary sources. Sulfuric acid is first emitted from vehicles as particles with a volume median diameter (VMD) of <0.1 μm (Stevens et al., 1976), which Gentry et al. (1982) define as ultrafine particles. These particles age by diffusion and impaction to a VMD of 0.20–0.30 μm. Although particles in this size range (<0.1–0.30 μm) deposit primarily in the alveolar region of the lung, there

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are reports that particle size has a significant influence on toxicity even when the particles have the same general region of deposition (Wolff et al., 1979; Amdur, 1958; Amdur et al., 1978). Ozone (O_3), a major environmental pollutant, has been widely studied both atmospherically and in health-related studies (U.S. Environmental Protection Agency, 1978). In the atmosphere, O_3 and H_2SO_4 mutually exist (Middleton, 1980; Stevens et al., 1978), excessively in highly populated areas and to a lesser degree in surburban and rural areas.

Recent studies of the interaction of O_3 and H_2SO_4 suggest the need for further research in this area. Gardner et al. (1977b) showed that a sequential exposure to O_3 followed by H_2SO_4 (0.25 μm VMD) significantly increased pneumonia-induced mortality rates in mice after a challenge with infectious bacteria; however, the reverse sequence, H_2SO_4 followed by O_3, did not affect the incidence of mortality. Grose et al. (1980) reported a significant decrease in ciliary beating frequency following a sequential O_3 and H_2SO_4 (0.25 μm VMD) exposure. Because of the results of sequential O_3 and H_2SO_4 exposures, the present study was performed to investigate the effects of a combined exposure to these pollutants.

This study investigates the effects of inhalation of ultrafine H_2SO_4 and simultaneous aerosol exposures to O_3 and H_2SO_4 on pulmonary host defense mechanisms. Ciliary beating frequency is representative of effects that are likely to occur in conducting airways, while host susceptibility to infection is thought to primarily reflect alveolar-level effects (Gardner and Graham, 1977).

METHODS

Simultaneous O_3 and H_2SO_4 Exposures

Female 30-d-old Cd-1 mice and male Syrian golden hamsters (Charles River Breeding Laboratories, Wilmington, Mass.) weighing 25 ± 3 g and 100 ± 10 g, respectively, were used. All exposures were conducted in a 0.33-m^3 stainless-steel Rochester-type inhalation exposure chamber (Hinners et al., 1968). Animals were separately exposed in wire-mesh, flow-through, stainless-steel cages. Deodorized animal cage board (UpJohn, Kalamazoo, Mich.) was placed in the bottom of the exposure chamber prior to each exposure to minimize the buildup of ammonia during exposures.

The chamber, generator, and air-monitoring systems are shown in Fig. 1. Filtered, dried, compressed air was split into generator and dilution streams. The generator stream was passed over the surface of heated (110–123°C) concentrated H_2SO_4 at a rate of 5 l/min. The H_2SO_4 vapors were swept out of the heated bottle through heated insulated lines and passed through a dry impinger to remove any large droplets that might be

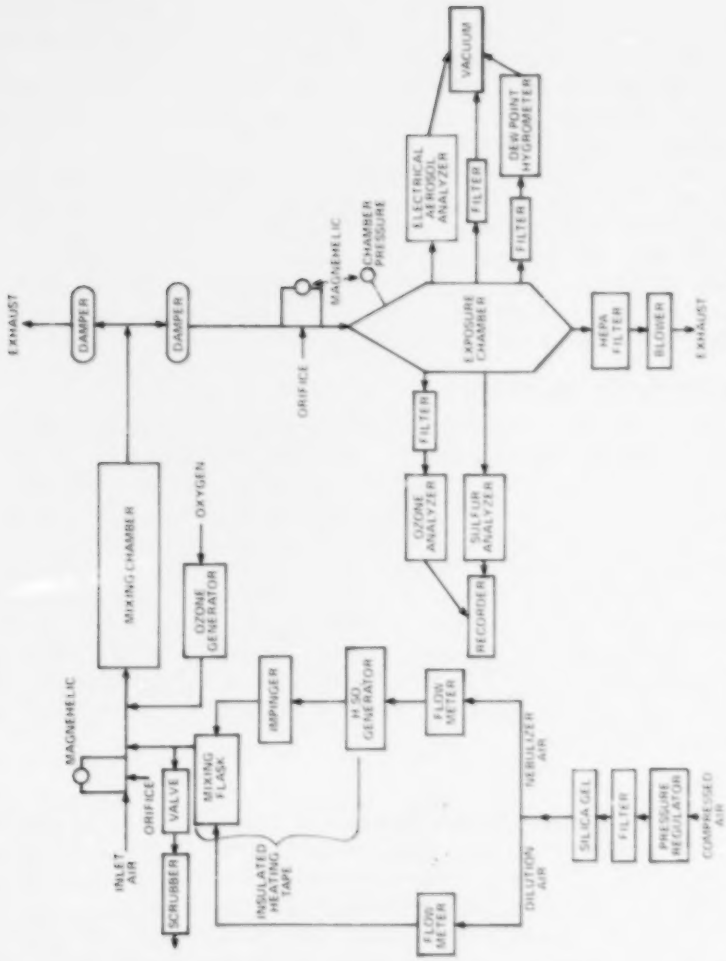

FIGURE 1. Sulfuric acid and O_3 exposure-system flow diagram.

present. This vapor-laden airstream then flowed into a three-neck flask and was mixed with dilution air. The dilution air was metered through a rotameter at 20 l/min. After mixing, the combined airstreams were injected into the chamber airstream. A bleed valve was installed to allow lower H_2SO_4 concentrations to be reached with minimal system changes. The chamber airflow was maintained at 1 volume change/min.

Ozone was generated by metering oxygen through a silent arc generator and was introduced into the exposure system just beyond the H_2SO_4 aerosol injection point (Fig. 1).

The combined H_2SO_4 and O_3 atmosphere passed through the mixing chamber and into the exposure chamber. The exposure chamber was maintained at a slight negative pressure to prevent any leakage of test atmosphere into the laboratory. The temperature and humidity were maintained at 24 ± 0.6°C and 33 ± 4%, respectively. After the exposure atmosphere flowed through the chamber, the exhaust was filtered by a HEPA filter and released to the atmosphere.

Ultrafine H_2SO_4 Exposures

All these exposures were conducted by placing animals in a Plexiglas head-only inhalation exposure chamber described by Adkins et al. (1980) (Fig. 2). The ultrafine H_2SO_4 generation system and the characteristics of the aerosol were described by Liu and Levi (1980). Ultrafine H_2SO_4-laden air from the generator was mixed with dried, conditioned chamber air in a mixing chamber and passed through the exposure chamber. Chamber exhaust was filtered by a HEPA filter and exhausted to the atmosphere. The chamber was maintained at a slight negative pressure to prevent leakage of H_2SO_4 into the exposure room. The mean chamber flow rate was maintained at 15.0 ft^3/min or approximately 2 air changes/min. The temperature and relative humidity were maintained at 74.7 ± 1.0°F and 30.5 ± 0.3%, respectively.

Monitoring

A total sulfur monitor (Meloy, Springfield, Va., model 160-2) provided an indication of the sulfur concentration during the simultaneous O_3 and H_2SO_4 exposures. The sample line was routed to minimize loss of the H_2SO_4 aerosol by impaction during transport of the sample. A sample was obtained from the chamber at a flow rate of 0.2 l/min. During the ultrafine H_2SO_4 exposures, a total sulfur analyzer (Monitor Labs, San Diego, Calif., model 8450) was used to obtain an estimate of the sulfur concentration. The analyzer continuously pulled a sample from the chamber at a flow rate of 0.18 l/min. An SO_2 permeation system (Nelson, 1976) was used to calibrate both analyzers biweekly.

Sulfate concentration for both chambers was measured by the method of Stevens et al. (1978), following collection on 1.0-μm Teflon-backed filters (Millipore Corporation, Bedford, Mass., catalog FALPO4700). An

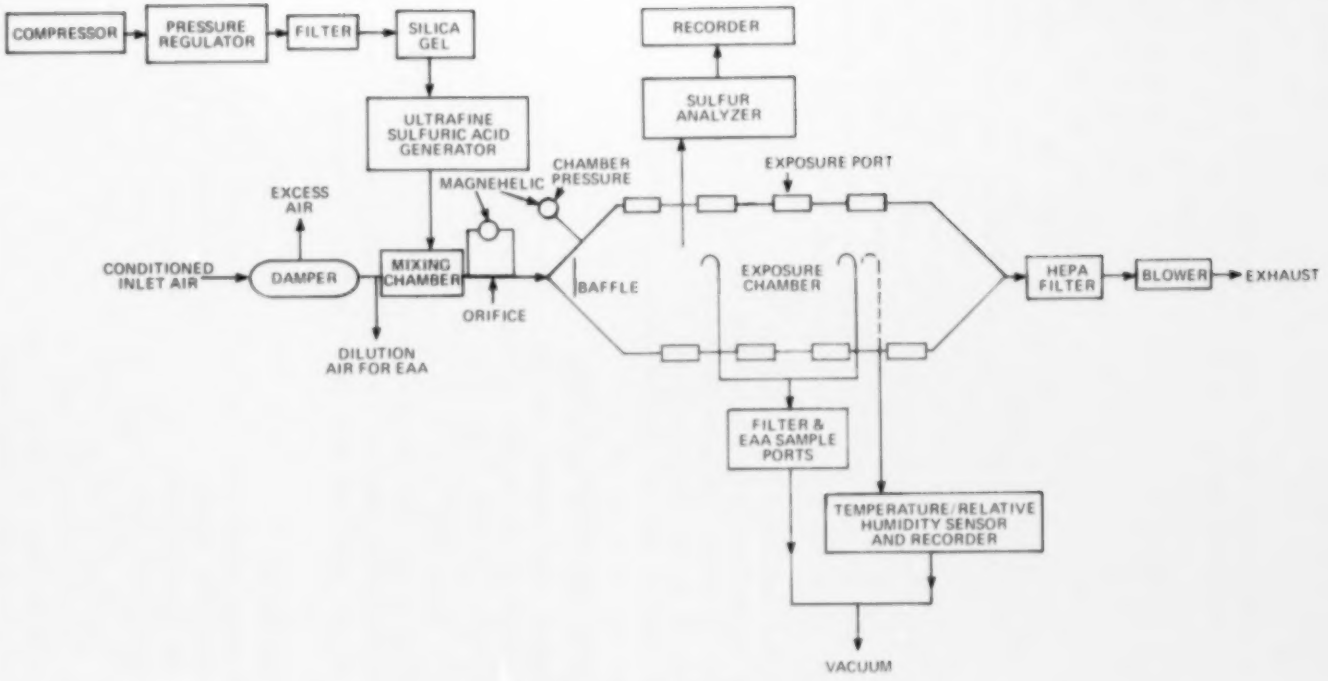

FIGURE 2. Ultrafine H_2SO_4 exposure-system flow diagram.

electrical aerosol analyzer (EAA) (Thermo Systems, Inc., St. Paul, Minn., model 3030) monitored chamber H_2SO_4 (0.01–1.0 μm) at a flow rate of 4.0 l/min every 30 min from the center of the chamber.

Ozone. A chemiluminescent O_3 analyzer (Bendix, Ronceverte, W. Va., model 8002) continuously monitored the O_3 concentration. Chamber air was drawn from the exposure chamber through a Teflon line and filtered to prevent the H_2SO_4 aerosol from contaminating the O_3 reaction chamber in the O_3 analyzer. During the study, the O_3 calibration procedure was updated to comply with the Environmental Protection Agency's promulgated method. The wet chemical method (*Federal Register*, 1971) was used for the first 15 exposures, and dynamic gas titration (*Federal Register*, 1976) was used for the remaining 13 exposures. When the wet chemical procedure was utilized, calibration of the O_3 analyzer was verified prior to each exposure. To establish zero and span points for the O_3 monitor, ambient air was pulled through a cartridge containing 8–14-mesh coconut charcoal. After a zero point was obtained, the charcoal cartridge was removed, and the concentration of O_3 in the chamber was allowed to stabilize. The O_3 concentration was then determined by the wet chemical procedure from an air sample drawn from the chamber. Generally, duplicate samples were taken (±10%), and the O_3 analyzer was set to the measured value. After updating the O_3 calibration to the gas titration procedure, multipoint calibrations were conducted biweekly.

Temperature and Relative Humidity

Temperature and relative humidity were continuously monitored during the simultaneous exposure using a dew-point hygrometer (EG and G International, Waltham, Mass., model 880). During the ultrafine acid exposures, a Rustrak sensor and recorder (Gulton Industries, Inc., Manchester, N.H., model 225) were used to monitor temperature and relative humidity (Fig. 2). A small vacuum pump pulled chamber air through a 25-mm Teflon-backed filter to remove the H_2SO_4 aerosol. The relative humidity of the chamber was determined by analyzing the filtered airstream.

Infectivity Model

The infectivity model has been previously described in detail (Gardner and Graham, 1977). Briefly, both air-control and pollutant-exposed mice were exposed simultaneously for 20 min to an aerosol of *Streptococcus pyogenes* immediately after the pollutant or filtered-air exposure. Following the infectious challenge, the animals were separated by treatment groups and housed in an isolation room for 15 d, during which time daily deaths were recorded. Mortality rates were expressed as percent difference between exposed and control animals. Relative mean survival time (RMST), comparing the average number of days the treated animals

survive as compared to the control animals, was also examined (Gardner et al., 1977a).

Ciliary Beating Frequency

The experimental procedure followed to assay tracheal ciliary beating frequency has been described by Grose et al. (1980). In brief, hamsters were studied immediately (0 h) and 72 h after exposure. After animals were anesthetized ip with sodium pentobarbital (100 mg/kg), they were exsanguinated through the abdominal aorta. Tracheas were excised and cut into individual rings. Ring cultures were held individually in culture medium (37°C) for 1 h until assayed. Ciliary beating frequency was microscopically determined immediately after surgery using stroboscopic illumination.

Statistical Analysis

To test the hypothesis of no treatment differences, univariate analysis of variance was used to analyze ciliary beating frequency data. Since experiments involved *in vivo* exposures, pretreatment beating frequency could not be determined. Hence, pollutant-exposed animals were compared to a group of animals exposed to clean air at each time point. The incidence of mortality, expressed as percent difference in mortality between exposed and control animals and RMST, were analyzed using Student's *t*-test.

RESULTS

Table 1 describes the details of the pollutant exposure conditions of this study. Exposure to a combined aerosol of 196 $\mu g\ O_3/m^3$ and 483 or 241 $\mu g\ H_2SO_4/m^3$ followed by a bacterial challenge resulted in a significant increase in percent mortality as compared to controls (Table 2). Those mice exposed to either 483 $\mu g\ H_2SO_4/m^3$ and 196 $\mu g\ O_3/m^3$ or to

TABLE 1. Descriptive Statistics for Various Exposure Conditions

	Concentration		Number of exposures	Temperature (°C)	Relative humidity (%)	H_2SO_4 VMD (μm)	H_2SO_4 sigma g
	O_3 (μg/m³)	H_2SO_4 (μg SO_4/m³)					
Simultaneous exposures	196 ± 12[a]	241 ± 28	12	24 ± 2.0	37 ± 6	0.20 ± 0.04	2.1 ± 0.1
	196 ± 8	483 ± 118	13	24 ± 1.8	34 ± 12	0.22 ± 0.04	2.1 ± 0.2
	196 ± 10	847 ± 195	3	23 ± 0.6	29 ± 4	0.27 ± 0.01	2.1 ± 0.1
Ultrafine exposures	–	365 ± 43	9	23 ± 0.9	31 ± 3	0.06 ± 0.01	2.3 ± 0.2
	–	458 ± 27	3	23 ± 1.2	30 ± 1	0.08 ± 0.01	2.2 ± 0.1
	–	543 ± 35	13	24 ±1.9	30 ± 7	0.08 ± 0.01	2.3 ± 0.2

[a]Mean ± standard deviation.

TABLE 2. Effect of Pollutant Exposure on Infectivity Model Parameters

Pollutant/exposures	Number of experiments (80 animals/ experiment)	% Difference in mortality (treated − control)[a]	Relative mean survival time (d) (treated − control)[a]
Simultaneous (3 h)			
483 μg H_2SO_4/m^3 + 196 μg O_3/m^3	13	7.5 ± 2.7[c]	0.6 ± 0.3[b]
241 μg H_2SO_4/m^3 + 196 μg O_3/m^3	12	6.0 ± 3.1[b]	0.6 ± 0.3[b]
Ultrafine (2 h/d)			
1 day × 543 μg H_2SO_4/m^3	13	4.3 ± 5.1	0.4 ± 0.5
5 day × 365 μg H_2SO_4/m^3	9	3.3 ± 2.2	0.3 ± 0.2

[a] Data are mean values ± the standard error of the mean.
[b] $p \leq 0.05$.
[c] $p \leq 0.01$.

241 μg H_2SO_4/m^3 and 196 μg O_3/m^3 died, on the average, 0.6 d sooner than control mice. Figure 3 illustrates the cumulative death rate based on percent over the 15-d holding period. Although the onset of the mortality was the same for all groups, the rate of mortality soon increased for the pollutant-exposed mice.

Ciliary beating frequency was measured after a 3-h simultaneous

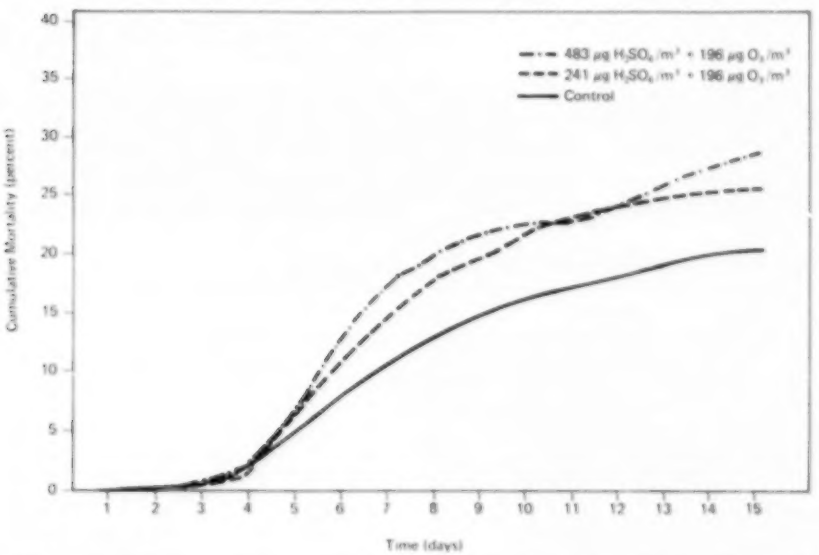

FIGURE 3. Temporal mortality patterns following challenge with infectious microorganisms for mice exposed to clean air or to H_2SO_4 and O_3.

TABLE 3. Ciliary Beating Frequency in Hamsters after Pollutant Exposure[a]

	847 μg H_2SO_4/m^3 + 196 μg O_3/m^3 × 3 h (Simultaneous exposure)	
Control[b]	0 h Postexposure	72 h Postexposure
957 ± 16 (15)	913 ± 16 (15)	926 ± 28 (15)
	458 μg H_2SO_4 × 2 h (Ultrafine H_2SO_4 exposure)	
1025 ± 20 (15)	958 ± 22 (19)	982 ± 25 (13)

[a] Data are mean values ± the standard error of the mean.
[b] Number of animals given in parentheses.

exposure to aged 847 μg H_2SO_4/m^3 and 196 μg O_3/m^3. Although there was a decrease in beating frequency at both 0 h and 72 h postexposure, it was not significantly lower than controls (Table 3). Further testing of this combination on ciliary beating frequency was therefore deemed unnecessary.

In addition to the simultaneous exposure to acid and O_3, animals were exposed to an aerosol of ultrafine H_2SO_4. An acute 2-h exposure caused no effect on mortality or RMST due to bacterial infection (Table 2). Therefore, the exposure length was increased to 2 h/d for 5 d. Percent mortality difference and RMST were still not significantly different from controls. This correlates with the upper-airway parameter of ciliary beating frequency. Immediately after a 2-h exposure to ultrafine H_2SO_4, and 72-h postexposure, ciliary beating frequency was slightly less than controls, but the decrease was not statistically significant (Table 3).

DISCUSSION

Because man exists in a complex chemical environment, there is a need to investigate possible additive, synergistic, and antagonistic effects of chemical combinations. The action of a sequential exposure to 196 μg O_3/m^3 and 1000 μg H_2SO_4/m^3 on incidence of mortality was reported by Gardner et al. (1977b) using the same infectivity model and exposure systems applied in the research reported here. In the research by Gardner et al., it was discovered that the order of the pollutant exposure determined the magnitude of the effect. They reported that neither O_3 nor H_2SO_4 alone at these concentrations caused any change in host susceptibility to infection. However, a sequential exposure to O_3 for 3 h followed by H_2SO_4 for 2 h caused a significant increase in mortality rates. When the order of exposure was reversed though—H_2SO_4 followed by

O_3—Gardner et al. reported no significant difference in mortality between treated animals and controls. Grose et al. (1980) exposed hamsters to 196 μg O_3/m^3 and 1000 μg H_2SO_4/m^3 alone and sequentially using techniques identical to those used in this study. Ciliary beating frequency was significantly decreased in animals exposed to H_2SO_4 alone but not O_3 alone. A sequential exposure to O_3 for 3 h followed by H_2SO_4 for 2 h resulted in an effect less than that observed with H_2SO_4 alone, but still significantly different from controls. This indicates a possible antagonistic effect between O_3 and H_2SO_4 on ciliary beating frequency. Further evidence of this antagonism may be implied by the results of this study, which show the simultaneous exposure to 196 μg O_3/m^3 and 847 μg H_2SO_4/m^3 had no significant effect on ciliary beating frequency as compared to controls. However, Last and Cross (1978) reported a significant synergistic increase in glycoprotein secretion by hamster tracheal organ explants after a combined 3-d exposure to 980 μg O_3/m^3 and 1000 μg H_2SO_4/m^3. Exposure to 980 μg O_3/m^3 alone decreased secretion rate, while exposure to 1000 μg H_2SO_4/m^3 alone caused no effect. The combined exposure, however, significantly increased glycoprotein secretion to 127% of that in controls. These results suggest that the combined effect of O_3 and H_2SO_4 on tracheal ciliary beating frequency may be due to an increase in glycoprotein secretions that protects the cilia, thus decreasing the deleterious effect of the acid on beating frequency. It may also indicate a chemical reaction of either pollutant with the glycoprotein secretions, decreasing the toxic effect previously reported.

The effect of the simultaneous O_3 and H_2SO_4 exposure in the alveolar region is illustrated by the infectivity results reported here. Exposure to a combination of either 483 or 241 μg H_2SO_4/m^3 with 196 μg O_3/m^3 resulted in statistically significant increased mortality in exposed mice as compared to controls. The actual mechanism of the infectivity model system has not been fully elucidated; however, numerous studies support the hypothesis that increased susceptibility is due to decreased clearance of bacteria. This may be a result of decreased phagocytic ability by damaged alveolar macrophages, as reported by Coffin and Gardner (1972). The increased susceptibility could also be due to O_3 damage in the alveolar area resulting in prolonged bacterial residence, reduction of clearance, or lung edema. Various studies supporting this concept have been reviewed by Gardner and Graham (1977).

Sulfuric acid aerosols of two different VMD values were used in this study. The 0.07-μm acid is representative of ultrafine H_2SO_4 as it first leaves the exhaust pipe of a vehicle. The 0.23-μm H_2SO_4 represents agglomerated particles. Thus, when individuals breathe a mist of ambient H_2SO_4, they are inhaling acid in a range from <0.1–0.30 μm. Normally, particles of either size would preferentially deposit in the gaseous exchange region of the lung.

Exposure to H_2SO_4 (0.23-μm VMD) or ultrafine H_2SO_4 alone does not affect the host's susceptibility to bacterial (Gardner et al., 1977b) or viral (Fenters et al., 1979) infection. Yet various studies have shown that acute exposure to H_2SO_4 with VMD ≥ 0.25 μm deleteriously affects either tracheal ciliary beating frequency or tracheal and bronchial particle clearance in hamsters (Grose et al., 1980; Schiff et al., 1979), donkeys (Schlesinger, 1978), and humans (Leikauf et al., 1981).

Even though an identical exposure to H_2SO_4 or O_3 alone does not affect susceptibility to infection, a combined O_3-H_2SO_4 exposure, whether it be simultaneous or sequential (Gardner et al., 1977b), does significantly increase mortality rate. The concentration of O_3 (196 $\mu g/m^3$) used in this study has been shown to increase mortality in the infectivity model if the technique is altered to include bacterial exposure during O_3 exposure; the O_3 exposure was for 3 h (Miller et al., 1978), as opposed to the 2 h used here. This concentration of O_3 has not been reported to cause other pulmonary alterations in mice after acute exposure, but due to the toxicity of O_3 (U.S. Environmental Protection Agency, 1978) it is conceivable that other subtle changes, undetectable by traditional techniques, occurred. Thus, it may be assumed that the O_3 exposure used in this study might have marginally compromised the mice to the extent that H_2SO_4 became effective. It is not possible to directly compare the results of the combined exposure to O_3 and H_2SO_4 in this study to a sequential O_3-H_2SO_4 exposure (Gardner et al., 1977b), since different concentrations of H_2SO_4 were used. However, since the sequential exposure—O_3 followed by H_2SO_4—did increase mortality, it can be suggested that O_3, in an as yet unknown fashion, potentiates the effects of H_2SO_4. Thus, it is possible that the combined exposure effects were also due to potentiation by O_3.

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